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ORIGINAL RESEARCH PAPER

Neferine inhibits LPS-ATP-induced endothelial cell pyroptosis via regulation of ROS/NLRP3/Caspase-1 signaling pathway

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Abstract

Background Oxidative stress-induced endothelial dysfunction and pyroptosis play an important role during chronic kidney disease (CKD) progression. Neferine, which is an alkaloid ingredient from the lotus seed embryo, has many biological actions such as anti-inflammatory, anticancer and antioxidant. However, the role of neferine in endothelial cell pyroptosis and the involved mechanism remain obscure. The aim is to probe the protective effects of neferine on cell pyroptosis and the involved underlying mechanism.

Methods After the HUVECs were primed with neferine treatment for 2 h prior to LPS and ATP exposure for 24 h, the cell proliferation was determined by BrdU; the cell LDH release was detected by LDH kits; the levels of intracellular ROS, MDA and SOD were tested by detection kits; Caspase-1 activity kit was used to determine caspase-1 activity; the contents of NLRP3, ASC, caspase-1, IL-1β, IL-18 and GSDMD were tested by RT-PCR and western blot.

Results We found that neferine could inhibit LPS-ATP-induced oxidative stress and the activation of NLRP3 inflammasome signaling, and increased the endothelial cell viability and SOD production. siRNA which mediated the knockdown of NLRP3 promoted the neferine-induced inhibition effects of cell pyroptosis. Furthermore, these neferine-induced effects were reversed by the over-expression of NLRP3.

Conclusions Our findings indicated neferine may reduce ROS by anti-oxidation and inhibit LPS-ATP-induced endothelial cell pyroptosis via blocking ROS/NLRP3/Caspase-1 signaling pathway, which provides the evidence for therapeutic effect in CKD.

Keywords Neferine · Cell pyroptosis · NLRP3 inflammasome · Chronic kidney disease (CKD) · ROS/NLRP3/Caspase-1 signaling pathway

Introduction

Chronic kidney disease (CKD) is a general term for heterogeneous disorders affecting the structure and function of kidney [1], accompanying by the cognitive dysfunction [2] and poor quality of life [3]. Vascular disease caused by endothelial cell injury is the main complication of CKD [4], and chronic inflammation and oxidative stress are critical for the development of CKD [5, 6]. The increasing evidences indicate the loss of some antioxidant substances (vitamins, zinc, selenium, etc.) and the damage mechanism of oxygen free radicals in the dialysis process of CKD patients cause strong oxidative stress [7–9]. In addition, CKD is associated with mitochondrial dysfunction that trigger an imbalance between the reactive oxygen species (ROS) and natural anti-oxidants that normally scavenge these pathological free radicals [10]. The clinical

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treatments can alleviate its pathological progression but the therapies are limited. Therefore, exploring the reduction of vascular endothelial cell damage caused by oxidative stress is of great significance for the prevention and treatment of CKD.

The NLRP3 inflammasome is an inflammatory protein complex and composed of the intracellular innate immune receptor NLRP3, the adaptor protein ASC and the protease caspase-1, which can help the body recognize endogenous and exogenous abnormal substances and release inflammatory factors IL-1β and IL-18 [11]. NLRP3 is one of the important signal receptors in the cells of innate immune that can be activated by a variety of pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP) [12]. The increasing studies have shown that NLRP3 inflammasome are enhanced in renal diseases such as diabetic nephropathy and uremia, and the contents of inflammatory factors such as IL-1β and IL-18 are significantly increased [13–15]. In addition, knocking down the NLRP3 gene or using the mice with NLRP3 knockout blocks the activation of NLRP3 inflammasome, thereby reducing kidney damage [16]. Therefore, the inhibition of NLRP3 inflammasome activation plays an important role in the prevention and treatment of immune diseases and inflammatory diseases.

Pyroptosis is a unique form of inflammatory cell death which is induced by inflammasome and is dependent on the activation of caspase-1 [17] which is responsible for the production of IL-1β and IL-18 [18]. The report finds that pyroptotic cell death contributes to inflammation and enhances the kidney damage [19]. More and more evidences show that combination with inhibitors of cell death pathways is likely to bring possible cure to patients suffering from kidney diseases [20–22]. Moreover, the activation of NLRP3 inflammasome and the resulting pyroptosis are involved in the mechanism of Cd-mediated human umbilical vein endothelial cells (HUVECs) [23]. The NLRP3 inflammasome activation-mediated cell pyroptosis may be critical for the development and progression of CKD.

Neferine, an alkaloid ingredient from the lotus seed embryo of Nelumbo nucifera, performs a variety of biological and pharmacological activities such as anti-tumor, anti-inflammatory, anti-oxidative, anti-fibrosis, and anti-arrhythmic [24, 25]. Neferine has showed therapeutic effects in several diseases through its anti-inflammatory and anti-oxidative activities and inhibition of cytokines [25]. Neferine which serves as a free radical scavenger enhances superoxide dismutase (SOD) activity and inhibits lipid peroxide (LPO) due to the hydroxyl group present in its structure [26]. ROS production plays an important role in the activation of NLRP3 inflammasome [27]. However, the role of neferine in the NLRP3 inflammasome activation and endothelial cell pyroptosis, and the involved mechanism remains obscure.

Taken together, we hypothesize that neferine reduces ROS by anti-oxidation and inhibits LPS-ATP-induced endothelial cell pyroptosis via blocking ROS/NLRP3/Caspase-1 signaling pathway. This study is to determine the effect of neferine on endothelial cell pyroptosis and the involved mechanism, which provides the evidence to treat CKD using neferine.

Materials and methods

Participant recruitment and ethical approval

All subjects were recruited from Xiangya hospital, Central South University. The serum samples were obtained from 20 patients with CKD stage 4–5 and healthy subjects. The vascular tissue was derived from AVF patients in hemodialysis center. Written informed consent was obtained from each subject and the study has been approved by the Ethics Committee of the Central South University. The ethics approval was given in compliance with the Declaration of Helsinki.

Cell culture and drug treatment

HUVECs cells were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). The HUVECs were cultured in DMEM supplemented with 1% (v/v) penicillin/streptomycin and 10% FBS and with a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Neferine (MCE, USA) was dissolved by DMSO with the 50 mM storage concentration. A dose gradient (0, 2, 4, 8, 16, 32 μM) was prepared by DMEM/F12 and administrated for 24, 48, and 72 h. LPS-ATP was selected to induce HUVECs cell pyroptosis for in vitro. Then HUVECs were primed with neferine treatment for 2 h prior to 1 μg/ml LPS (Sigma, USA) and 5 mM ATP (Sigma, USA) exposure. Cells cultured without any exposure and treatment were used as a control.

siRNA, vector construction and cell transfection

HUVECs were transfected with either 10 mM NLRP3-targeting siRNA (Santa Cruz) or a control nonspecific siRNA (Santa Cruz) using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). NLRP3 gene cDNA was cloned into the pcDNA3.1 plasmid vector.

Cell viability assay

Cell viability was tested using the Cell Counting Kit-8 (CCK-8) (Dojindo Kumamoto, Japan) as described by the manufacturer. In brief, HUVECs were cultured to reach the desired confluence in 96-well plates. Then, HUVECs were incubated with different treatment for 2 h at 37 °C, adding CCK-8 to each well. Following incubation, a microplate

reader (Tecan, Mannedorf, Switzerland) with an excitation wavelength of 488 nm was used to detect the absorbance.

5-Bromodeoxyuridine (BrdU) detection

The cell proliferation was detected by the labeling 5-bromodeoxyuridine (BrdU) detection. Briefly, BrdU was labeled using as one of these moieties and incorporating into the DNA of proliferating cells to study cell viability, and detected by immunofluorescence according to the manufacturer's protocol.

Flow cytometry

Flow cytometric was used to test pyroptosis by propidium iodide (PI) and FAM-FLICA in vitro Caspase-1 Detection Kit (ImmunoChemistry Technologie, Bloomington, Minnesota, USA) according to the manufacturer's instructions. In brief, the cells were harvested and incubated with red caspase-1 detection probe (FLICA 660-YVAD-FMK) at 37 °C in the dark for 60 min after treatment. At the end of incubation, the unbound FLICA reagent was washed away using cellular wash buffer. HUVECs were then stained with PI for a further 10 min at 37 °C protecting from light. HUVECs were then analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA). Pyroptotic cells were defined as double positive for FLICA 660-YVAD-FMK and PI.

Lactate dehydrogenase (LDH) release assay

After HUVECs had been exposed to the various treatments, the culture supernatants were harvested, and the levels of LDH were determined using the LDH cytotoxicity detection kit (Roche, Basel, Switzerland) as described by the manufacturer. The microplate reader (Tecan) with an excitation wavelength of 490 nm was used to measure the absorbance of samples.

Enzyme-linked immunosorbent assay (ELISA)

The levels of inflammatory mediators including IL-1β and IL-18 in the serum samples were measured using enzyme-linked immunosorbent assay (ELISA) in accordance with the protocols from the IL-1β and IL-18 kits (NeoBioscience, China).

Detection of reactive oxygen species (ROS)

ROS production in vascular tissues or HUVECs cells were measured using microplate reader or fluorescence microscopy using 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) as fluorescent probes following the manufacturer's protocols.

Detection of malondialdehyde (MDA) and superoxide dismutase (SOD)

Measurement of superoxide dismutase (SOD) and malondialdehyde (MDA) level was performed using commercial assay Kits (Cayman Chemical Company) as described by the manufacturer.

RNA extraction and real-time PCR

Total RNA of HUVECs cells was extracted using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The concentrations of RNA were measured by a NanoDrop 2000 spectrophotometer (Takara, China). The total RNA was reversed transcribed into synthesis cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The RT-PCR reactions were performed using the CFX96TM Real-time System (Bio-Rad, Hercules, CA, USA) with the SYBR Premix Ex Taq II Kit (Takara, Japan). Finally, the threshold cycle (Ct) was determined and the relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as internal controls. The sequences of the primers used are provided in Supplementary Table 1.

Immunofluorescence

Immunofluorescence staining was performed to detect the expression of NLRP3 and caspase-1 in HUVECs, which was imaged by a fluorescence microscope (Leica, Germany).

Protein extraction and western blot

Cells cultured in six-well plates were collected and lysed with RIPA lysis buffer (Beotime, Shanghai, China). The concentrations of protein were determined using a BCA protein assay kit (Bio-Rad, Mississauga, ON, Canada). Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore), and incubated with primary antibodies: anti-NLRP3 antibody (1:1000, Abcam, England), cleaved caspase-1 antibody (1:800, Proteintech, USA), cleaved ASC antibody (1:1000, Sigma, USA), cleaved IL-1β antibody (1:1000, Cell Signaling Technology, USA), cleaved IL-18 antibody (1:1000, Cell Signaling Technology, USA), cleaved GSDMD antibody (1:800, Proteintech, USA), anti-β-actin antibody (1:1000, Sigma, USA). The membrane was washed with TBST containing 0.1% Tween 20 for three times. And then membrane was incubated with HRP-labeled goat anti-mouse/rabbit IgG (1:5000, Sigma, USA) for 1 h at room temperature. Finally, the membranes were washed and exposed to ECL (Millipore) substrate and

visualized using the chemiluminescence detection system (Biorad). The intensity of the bands was quantified using ImageJ software tools.

Statistical analysis

All data, which were obtained from at least three independent experiments, were performed as the mean ± standard deviation (SD). The differences of variables among groups were tested using one-way analysis of variance (ANOVA), followed by the LSD post hoc test for multiple comparisons. All statistical analyses were used by Graphpad Prim 5 (GraphPad Software, La Jolla, CA, USA). The value of $p < 0.05$ in a two-side was considered statistically significant.

Results

The activation of NLRP3 inflammasome in the patients with CKD

First, we determined NLRP3 inflammasome activation in the patients with CKD. The maturation of IL-1β and IL-18 and the cleavage of caspase-1, ASC and NLRP3 are the hallmarks of NLRP3 inflammasome activation, thus we measured the levels of these indicators to examine the activation of NLRP3 inflammasome. As shown in Fig. 1a, b, the levels of IL-1β and IL-18 in the serum samples of the CKD patients were significantly higher than those in the controls. In addition, compared with the controls, the expressions of NLRP3, ASC, caspase-1 and GSDMD which were detected by immunohistochemistry were significantly up-regulated in the CKD patients (Fig. 1c). Consistently, similar changes of NLRP3, ASC, C-caspase1 and GSDMD-N which were detected by western blot were observed in vascular tissue of CKD patients (Fig. 1d). The level of ROS in vascular tissues of the CKD patients was significantly higher than that in the controls (Fig. 1e). These results indicated that the level of ROS was increased and NLRP3 inflammasome was activated in blood and vascular tissues of CKD patients, thereby inducing the increased expression of inflammatory factors and the development of cell pyroptosis.

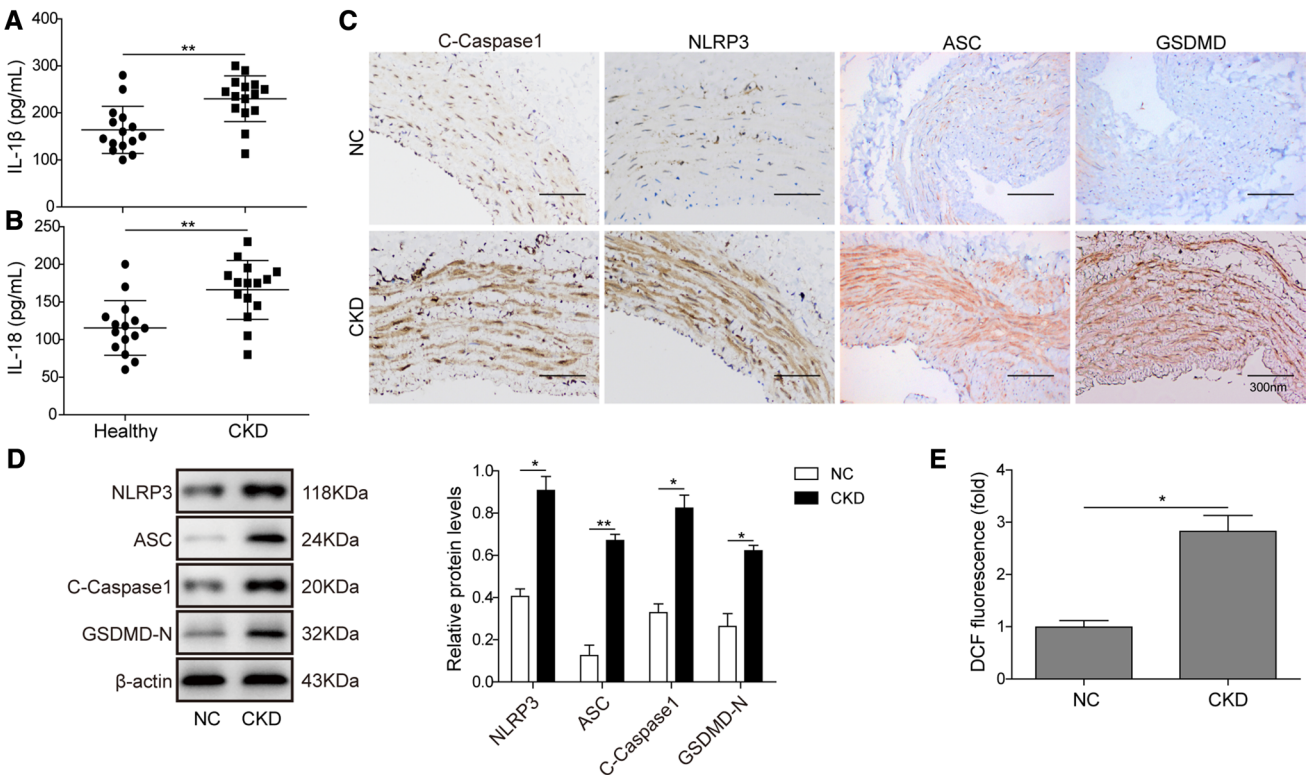


Fig. 1 The activated NLRP3 inflammasome induced the increased expression of inflammatory mediators and cell pyroptosis in the patients with CKD. **a**, **b** The levels of IL-1β and IL-18 in the serum samples were determined by ELISA assay. The data are performed by mean ± SD. **c** The expression of NLRP3, ASC, C-caspase1 and GSDMD in vascular tissue were detected by immunohistochemistry. **d** The protein expression of NLRP3, ASC, C-caspase1, GSDMD-N and β-actin in vascular tissue were detected by western blot. **e** The level of ROS in vascular tissue was detected by the fluorescent probe carboxy-H_2DCFDA. $n = 15$ in each group. *$p < 0.05$, **$p < 0.01$

Neferine suppressed LPS-ATP-induced cell pyroptosis and functional impairment

Initially, we explore the effects of different concentrations of neferine (0, 2, 4, 8, 16 and 32 μM) on the viability of HUVECs cells. According to MTT assay results, 2 μM neferine was selected as the most optimal concentration and used in the following study (Supplementary Fig. 1). To elucidate the association between neferine and cell pyroptosis, LPS-ATP was used to induce HUVECs cell pyroptosis for in vitro experiments. The group was as follows: (1) control group; (2) LPS-ATP group; (3) neferine pre-treatment group. Finally, the effects of neferine pre-treatment on cell proliferation and cell pyroptosis were estimated. As shown in Fig. 2a–d, compared with the controls, the LPS-ATP exposure led to a significant decrease of the cell viability, cell proliferation and cell angiogenic ability, and a significant increase of the cell pyroptosis. While, neferine pre-treatment obviously suppressed LPS-ATP-induced cell pyroptosis and functional impairment. In addition, the levels of LDH and C-caspase1 protein in the LPS-ATP exposure were significantly higher than those in the controls and neferine pre-treatment. The results indicated that LPS-ATP could induce cell pyroptosis and functional impairment mediated by caspase-1 activation, while neferine pre-treatment obviously suppressed LPS-ATP-induced cell pyroptosis and functional impairment.

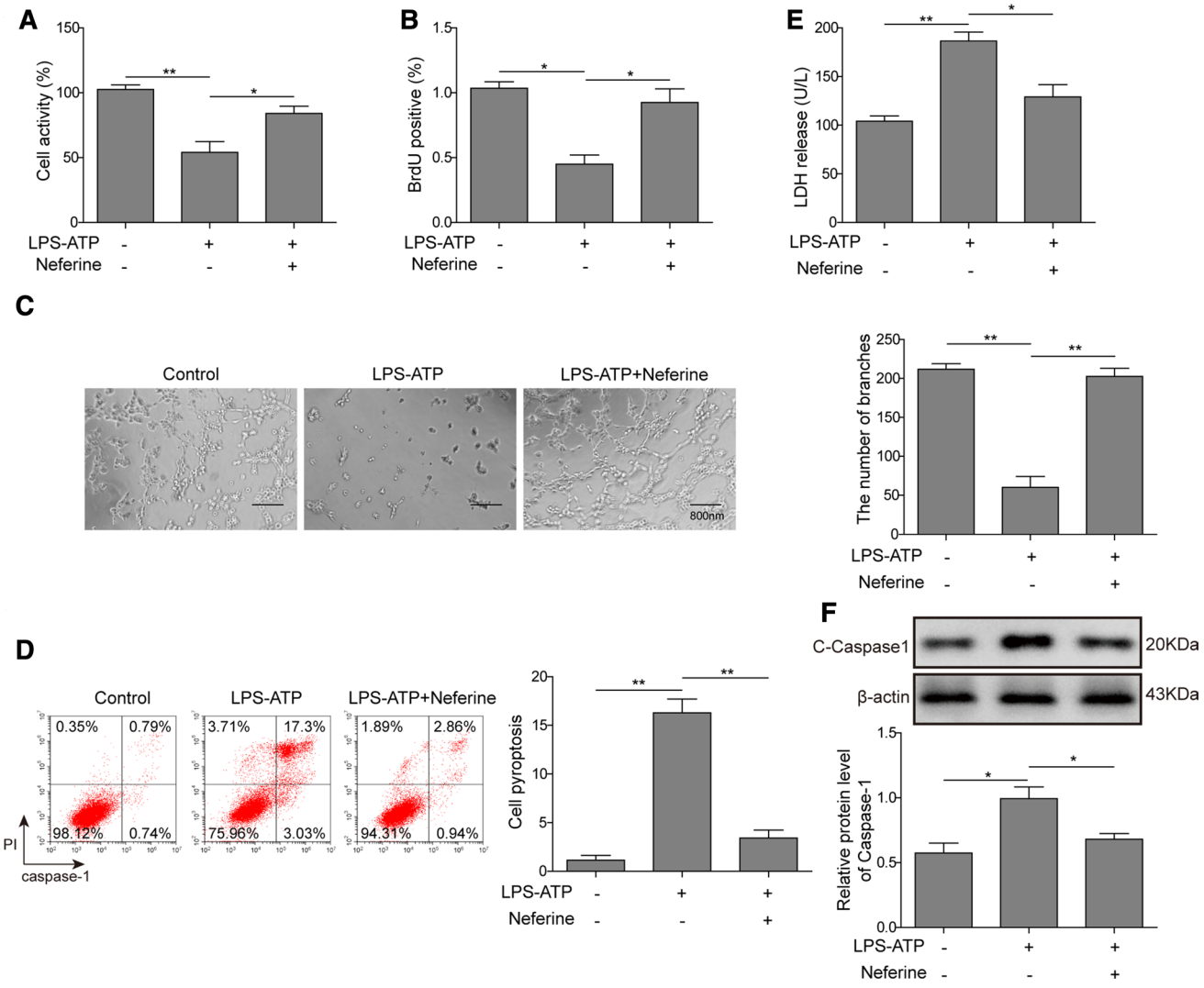


Fig. 2 Neferine suppressed LPS-ATP-induced cell pyroptosis and functional damage by promoting cell proliferation. HUVEC cells were pre-treated with 2 μM neferine for 2 h before primed with 1 μg/ml LPS for 4 h followed by stimulation with 5 mM ATP for 30 min. **a** The cell viability was detected by CCK8. **b** The cell proliferation was detected by BrdU. **c** The angiogenesis was detected by angiogenesis experiment. **d** The cell pyroptosis was detected by flow cytometry. **e** The cell LDH release was detected by LDH kit. **f** The protein expression of C-caspase1 was detected by western blot. $*p<0.05$, $**p<0.01$

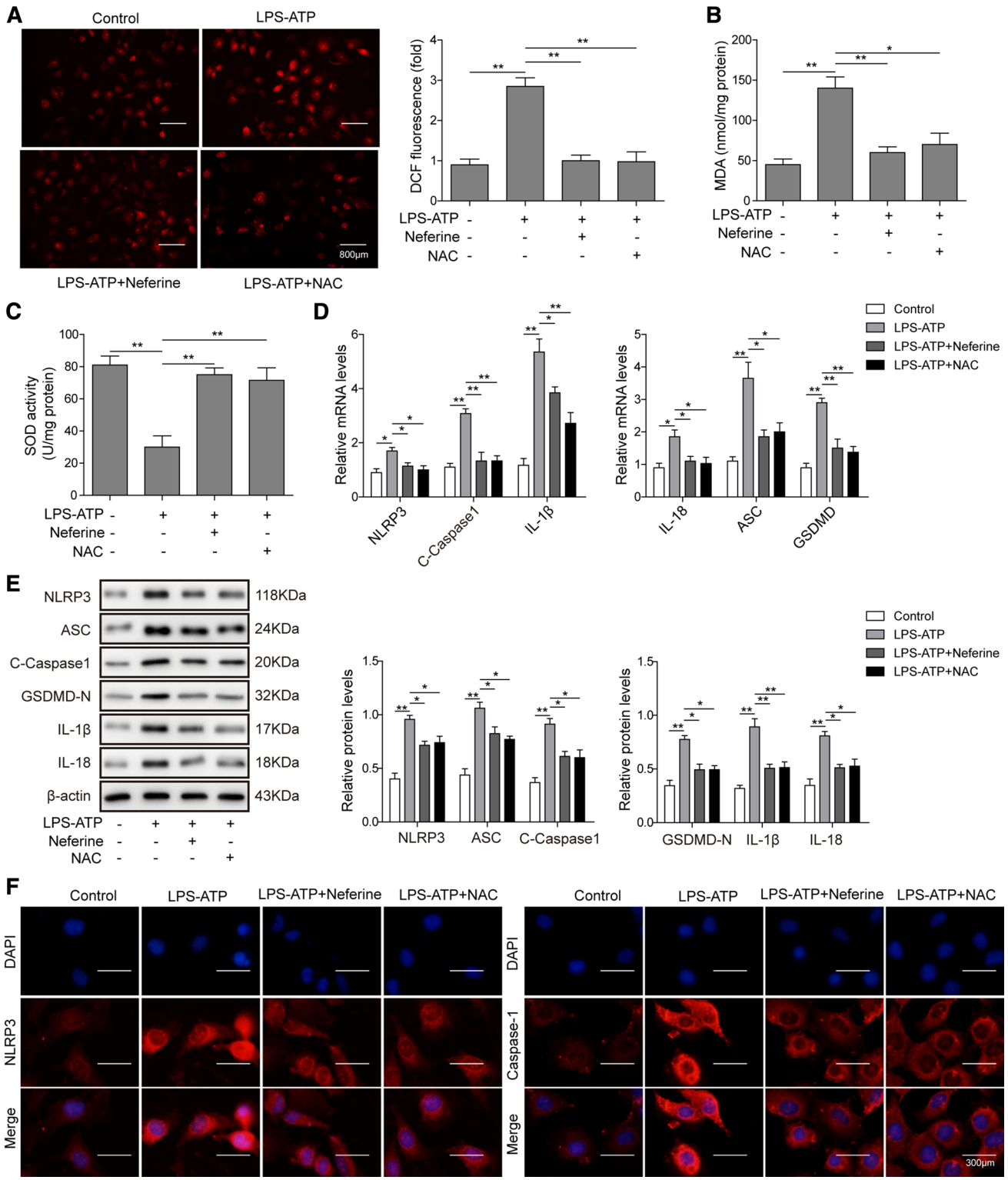


Fig. 3 Neferine acts as a ROS scavenger, inhibiting oxidative stress and inflammatory response. HUVEC cells were pre-treated with 2 μM neferine or 10 mM NAC for 2 h before primed with LPS-ATP. **a**, **c** The ROS, MDA and SOD contents were determined by detection kits. **d** The mRNA levels of inflammatory mediators including NLRP3, caspase-1, ASC, GSDMD, IL-1β, and IL-18 were detected by RT-PCR. **e** The levels of pyroptosis-related protein including NLRP3, ASC, C-caspase1, GSDMD-N, IL-1β, and IL-18 was detected by western blot. **f** The expressions of NLRP3 and C-caspase1 were detected by immunofluorescence. $*p < 0.05$, $**p < 0.01$

Neferine acts as a ROS scavenger, inhibiting oxidative stress and inflammatory response

To further explore the role of neferine in the regulation of LPS-ATP-induced cell pyroptosis by anti-oxidation action, we used the ROS scavenger (NAC) as a control to verify the effect of neferine on oxidative stress and inflammatory response induced by LPS-ATP. As shown in Fig. 3a–c, compared with the controls, the LPS-ATP exposure led to the oxidative stress of cell, with a significant increase of ROS and MDA production, and a significant decrease of SOD production. While neferine treatment obviously suppressed LPS-ATP-induced oxidative stress, and the effect was not significantly different from NAC treatment. We further investigated the influence of neferine on NLRP3 inflammasome activation and inflammatory factor. The mRNA levels of inflammatory mediators including NLRP3, caspase-1, ASC, GSDMD, IL-1β and IL-18 in the LPS-ATP exposure were significantly higher than those in the controls. While neferine treatment obviously suppressed LPS-ATP-induced inflammatory factor, and the effect was not significantly different from NAC treatment (Fig. 3d). As shown in Fig. 3e, the levels of pyroptosis-related protein including NLRP3, C-caspase1, ASC, GSDMD-N, IL-1β and IL-18 changed consistently. In addition, the expressions of NLRP3 and C-caspase1 which were detected by immunofluorescence got the same results (Fig. 3f). The results suggested that the suppression of ROS by neferine contributes to the prevention of NLRP3 activation and inflammatory factor release.

Neferine inhibits NLRP3 activation and inflammatory factor release by reducing ROS production

In addition, we used the ROS inducer (500 μM $CoCl_2$) as a control to verify the effect of neferine on anti-oxidation

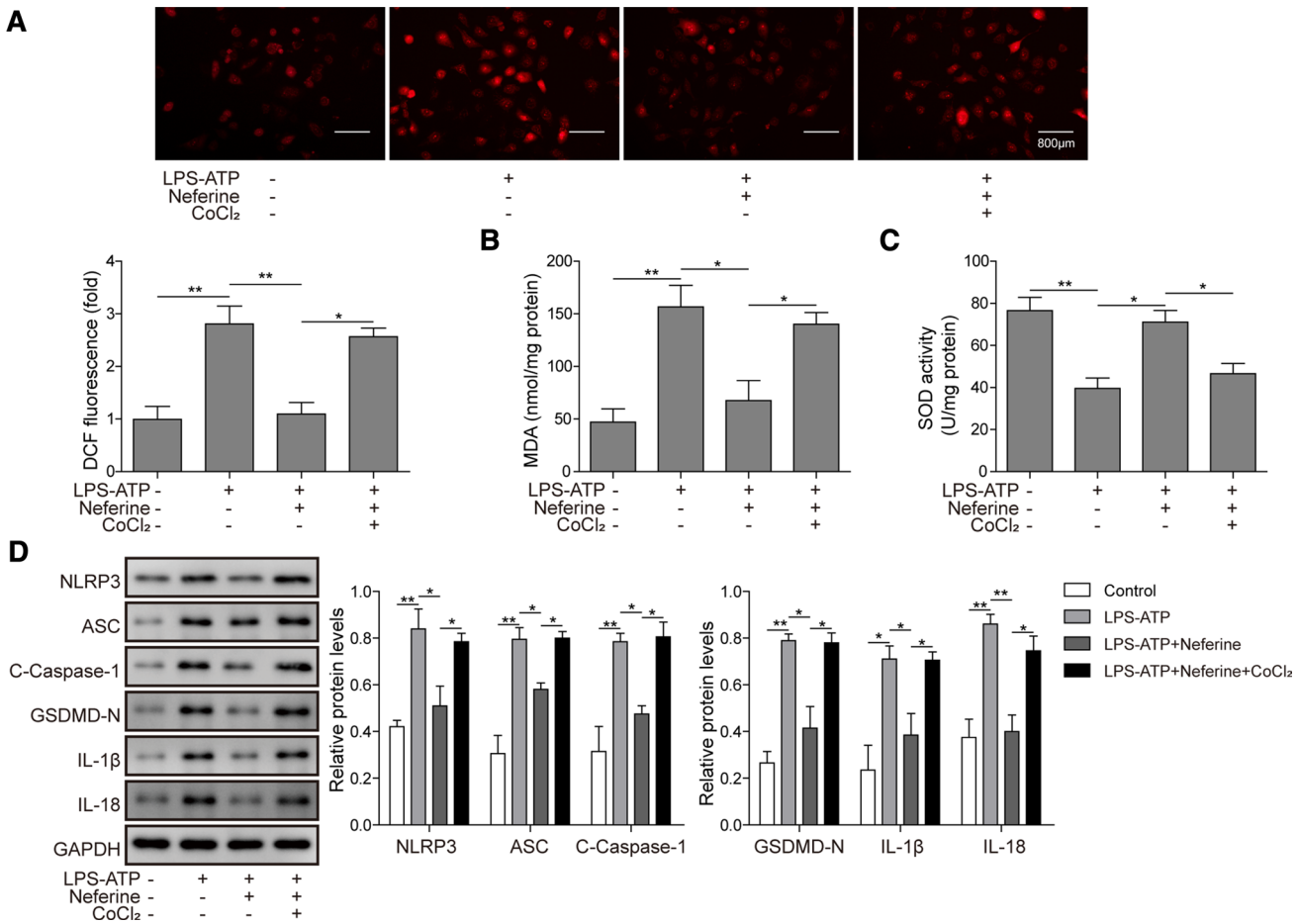


Fig. 4 Neferine inhibits NLRP3 activation and inflammatory factor release by reducing ROS production. HUVEC cells were pre-treated with 2 μM neferine or 500 μM $CoCl_2$ for 2 h before primed with LPS-ATP. **a**, **c** The ROS, MDA and SOD contents were determined by detection kits. **d** The levels of pyroptosis-related protein including NLRP3, ASC, C-caspase1, GSDMD-N, IL-1β, and IL-18 were detected by western blot. $^{*}p < 0.05$, $^{**}p < 0.01$

action to regulate NLRP3 activation induced by LPS-ATP. As shown in Fig. 4a–c, compared with the controls, the LPS-ATP exposure led to the oxidative stress of cell, with a significant increase of ROS and MDA production, and a significant decrease of SOD production. While neferine treatment obviously suppressed LPS-ATP-induced oxidative stress, and the ROS inducer of $CoCl_2$ significantly increased the production of ROS and MDA, and significantly decreased the production of SOD. The levels of pyroptosis-related protein including NLRP3, C-caspase1, ASC, GSDMD-N, IL-1β and IL-18 in the LPS-ATP exposure were significantly higher than those in the controls. While neferine treatment obviously suppressed LPS-ATP-induced inflammatory factor, and the ROS inducer of $CoCl_2$ significantly increased the levels of pyroptosis-related protein (Fig. 4d).

The changes of NLRP3 inflammasome influenced the effects of neferine on LPS-ATP-induced cell pyroptosis

Since neferine functioned like a ROS scavenger, it inhibited NLRP3 activation and inflammatory factors release by reducing ROS production. We further explored the effects of NLRP3 on neferine function by silencing or overexpressing NLRP3. Pre-treatment with neferine significantly inhibited LPS-ATP-induced ROS and MDA production, this effect was significantly enhanced by silencing NLRP3, while this effect was reversed after overexpressing NLRP3 (Figs. 5a, b; 6a, b). In addition, since LPS-ATP reduced the production of SOD, pre-treatment with neferine significantly increased the production of SOD, which was significantly enhanced after silencing NLRP3 while was reversed after overexpressing NLRP3 at the same time (Figs. 5c, 6c). As shown in Figs. 5d–f and 6d–f, silencing of NLRP3 inflammasome significantly inhibited LPS-ATP-induced cell pyroptosis and the expression of LDH and pyroptosis-related protein including NLRP3, C-caspase1, ASC, GSDMD-N, IL-1β and IL-18, while the effect was reversed after overexpressing NLRP3. In addition, the expressions of NLRP3 and C-caspase1 which were detected by immunofluorescence got the same results (Figs. 5g, 6g). The results indicated that the changes of NLRP3 inflammasome influenced the effects of neferine on LPS-ATP-induced cell pyroptosis and inflammatory factor release by regulating oxidative stress.

Discussion

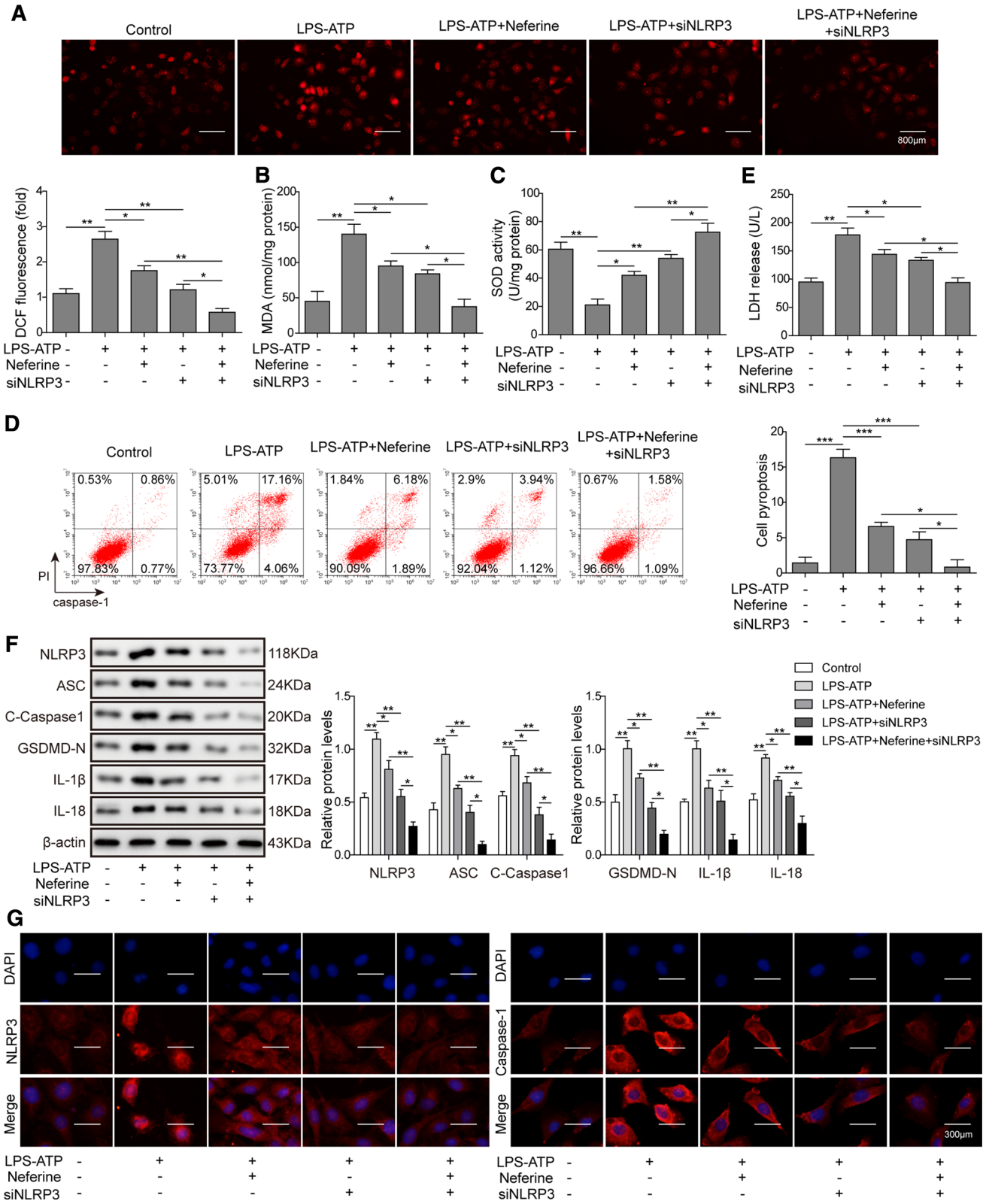
CKD, which performs a high morbidity and mortality, brings serious social and economic burdens [1]. However, the treatment of CKD still remains a clinical challenge. The increasing evidences show that inflammatory processes are crucial for the pathogenesis and progression of CKD [10]. The

Fig. 5 The silenced NLRP3 inflammasome promoted the resistance of neferine for LPS-ATP-induced cell pyroptosis. HUVECs were transfected with NLRP3-targeting siRNA before stimulated by LPS-ATP alone or treated with 2 μM neferine following LPS-ATP. **a**, **c** The ROS, MDA and SOD contents were determined by detection kits. **d** The cell pyroptosis was detected by flow cytometry. **e** The cell LDH release was detected by LDH kit. **f** The levels of pyroptosis-related protein including NLRP3, ASC, C-caspase1, GSDMD-N, IL-1β, and IL-18 were detected by western blotting. **g** The expression of NLRP3 and C-caspase1 was detected by immunofluorescence. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

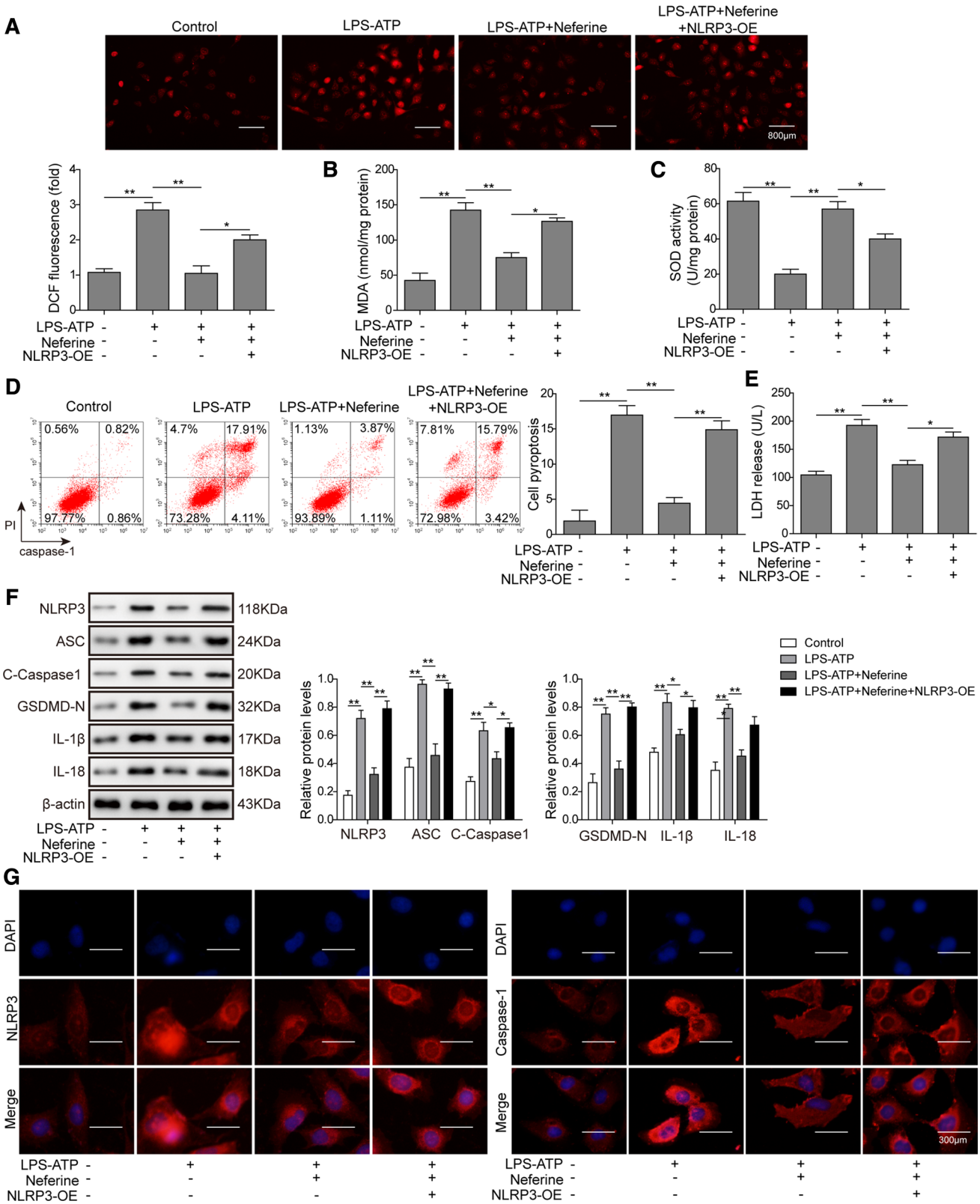
results showed that NLRP3 inflammasome was activated in blood and vascular tissues of CKD patients which was activated by oxidative stress, inducing the increased expression of inflammatory factors and the development of cell pyroptosis. The results indicated that the new drugs which reduce vascular endothelial cell pyroptosis caused by oxidative stress are an important therapeutic approach for CKD and its complications.

In our present study, we utilized LPS and ATP to stimulate HUVECs inducing the endothelial cell pyroptosis. Considering the important role of ROS in activating inflammasome, we detected the role of oxidative stress in endothelial cell pyroptosis. Consistently with previous studies [10, 27], ROS production played an important role in the activation of NLRP3 inflammasome since ROS scavenger (NAC) prevented the release of inflammatory factors and activation of NLRP3 inflammasome. In addition, our results confirmed that the activated calpain releases a pool of caspase-1 sequestered by the cytoskeleton to regulate NLRP3 activation which induced cell pyroptosis [17, 18]. Taken together, the results indicated that LPS-ATP induced endothelial cell pyroptosis via regulation of ROS/NLRP3/Caspase-1 signaling pathway.

Neferine has exhibited various biological activities such as antioxidant, anti-inflammatory, anticancer, and antibacterial effects [26, 28, 29]. Thus, neferine has been widely used as agent in the prevention of various cancers, inflammatory disorders, and hypertension [30–33]. More and more evidences show that neferine inhibits the release of cytokines and vascular endothelial cell injury, due to its strong radical scavenging activity [26, 33]. Our results indicated that the pre-treatment of neferine could suppress LPS-ATP-induced HUVECs pyroptosis and inhibited the augment of ROS in LPS-ATP-treated HUVECs. The low levels of SOD and MDA in LPS-ATP-treated HUVECs were also prevented by neferine. While neferine treatment obviously suppressed LPS-ATP-induced oxidative stress, and the effect was not significantly different from NAC treatment. The results suggested that neferine which serves as a free radical scavenger, markedly inhibited LPS-ATP-induced activation of ROS/NLRP3/Caspase-1 signaling pathway in HUVECs.



The NLRP3 inflammasomes have been linked to some autoinflammatory and autoimmune diseases [34]. The role of NLRP3 inflammasomes in endothelial cell pyroptosis suggests that inhibition of NLRP3 inflammasomes activation is important for the prevention and treatment of inflammatory diseases [35]. In our present study, neferine is crucial


A
Control
LPS-ATP
LPS-ATP+Neferine
LPS-ATP+Neferine +NLRP3-OE
800μm
DCF fluorescence (fold)
B
MDA (nmol/mg protein)
C
SOD activity (U/mg protein)
LPS-ATP
Neferine
NLRP3-OE
D
Control
LPS-ATP
LPS-ATP+Neferine
LPS-ATP+Neferine +NLRP3-OE
0.56%
0.82%
97.77%
0.86%
4.7%
17.91%
73.28%
4.11%
1.13%
3.87%
93.89%
1.11%
7.81%
15.79%
72.98%
3.42%
PI
caspase-1
Cell pyroptosis
E
LDH release (U/L)
F
NLRP3
ASC
C-Caspase1
GSDMD-N
IL-1β
IL-18
β-actin
118KDa
24KDa
20KDa
32KDa
17KDa
18KDa
43KDa
Relative protein levels
Control
LPS-ATP
LPS-ATP+Neferine
LPS-ATP+Neferine+NLRP3-OE
G
DAPI
NLRP3
Merge
Caspase-1
300μm

◂**Fig. 6** The activated NLRP3 inflammasome inhibited the resistance of neferine for LPS-ATP-induced cell pyroptosis. NLRP3-overexpressing plasmid was infected by HUVEC cells for 48 h, then cells were treated with 2 μM neferine following LPS-ATP. **a**, **c** The ROS, MDA and SOD contents were determined by detection kits. **d** The cell pyroptosis was detected by flow cytometry. **e** The cell lactate dehydrogenase release was detected by LDH kit. **f** The levels of pyroptosis-related protein including NLRP3, ASC, C-caspase1, GSDMD-N, IL-1β, and IL-18 were detected by western blot. **g** The expression of NLRP3 and C-caspase1 was detected by immunofluorescence. $*p < 0.05$, $**p < 0.01$

for the development of endothelial cell pyroptosis by inhibiting the activation of NLRP3 inflammasomes. Moreover, we further showed that the changes of NLRP3 inflammasome influenced the effects of neferine on LPS-ATP-induced cell pyroptosis. The data presented in the current study showed the silence of NLRP3 inflammasome or the reduction of ROS production which inhibited the LPS-ATP-induced caspase-1 activation, inflammatory cytokines (IL-1β and IL-18) secretion, and endothelial cell pyroptosis. Furthermore, the activation of NLRP3 inflammasome and the resulting pyroptosis suggest that inhibition of NLRP3 inflammasome activation was important for the prevention and treatment of CKD.

In summary, neferine suppressed the pyroptosis of endothelial cells via decreasing oxidative stress and release of inflammatory factors, inactivating ROS/NLRP3/Caspase-1 signaling pathway. This study suggests neferine as a potential therapeutic agent for endothelial dysfunction in CKD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study. We would like to give our sincere gratitude to the reviewers for their constructive comments.

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